# Analytical Methods for Determining Pectin Composition

Chemical and modern chromatographic methods have been applied to the determination of pectin composition and structure. The dominant structural feature of pectin is a linear chain of galacturonic acid residues, some of which are esterified with methyl groups. Chemical methods (including reactions with carbazole and substituted phenols) and chromatographic methods (GLC and HPLC) are available for galacturonic acid determination. Methyl ester levels are determined either chemically (after oxidation to formaldehyde) or by GLC after pectin ester saponification. A variety of neutral sugars are present in pectin, mainly rhamnose, galactose, arabinose, and xylose. Effective GLC procedures are available for their determination, and applicable liquid chromatographic methods have been developed. Measurements of less the less-common substituents, such as O-acetyl and O-feruloyl esters have been achieved by colorimetric and titrimetric procedures. Developments in infrared and $^{13}$C-NMR spectroscopy have resulted in these being applied to structural analysis in pectin.

Pectin polysaccharides and the hemicelluloses are matrix components in the cell walls of higher plants. Traditionally, these classes of carbohydrates have been defined operationally by their presence in fractions obtained by sequential extraction of cell walls. The pectic substances are extracted with water, dilute acid, or with calcium chelating agents, such as EDTA, ammonium oxalate, or sodium hexametaphosphate. But classification of polysaccharides is best based on structural components rather than on the method used for its isolation. According to structure, the pectic substances would include galacturonans, rhamnogalacturonans, arabinans, galactans, and arabinogalactans which possess a linear β-1,4-D -galactan backbone.

A recent classification (1) describes the pectic polysaccharides as those polymers found in covalent association with galacturonosyl-containing polysaccharides. The hemicelluloses are those carbohydrate polymers which are noncovalently associated with cellulose. Diverse categories of pectic polysaccharides occur not only among plant sources, but among tissues in a given source.

A comprehensive (2) review on the structure of pectin has recently been published. Pectin was described as consisting of a branched block, in which the main galacturonan chain is interrupted by rhamnose units. Many of these rhamnoses carry arabinan or galactan chains; the galactan chains are sometimes further substituted with arabinan segments. These heavily branched galacturonan chains alternate with unbranched blocks in which rhamnoses are rarely present. Methyl esters of galacturonic acid are also present as blocks, alternating with sequences of non-esterified galacturonic acid.

This review will describe the analytical methods available to determine the structural components of pectin. These features determine the important physical, chemical, and biological properties of pectin. Included will be discussion of galacturonic acid determinations, degree of esterification with methyl groups, the neutral-sugar composition, and analysis of some less-common entities, such as O-acetyl and O-feruloyl linkages.

## Quantitative Analysis of Galacturonic Acid in Pectin

The dominant and unifying structural feature in pectins is a linear 1→4-α-linked D-galactopyranosyl-uronic acid chain. α-L-Rhamnosyl residues are inserted at intervals in the chain, and variable proportions of the uronic acid residues are esterified with methanol. Neutral sugars other than rhamnose are present, and neutral sugar levels often total about 20%. The other neutral sugars are mainly D-galactose, L-arabinose, and D-xylose, and these are likely to be attached in branches to the rhamnose residues in the main chain. In a later section, various chromatographic approaches for determining the levels of individual neutral sugars will be described.

Chemical Methods. Determinations of galacturonic acid of pectin usually includes both the free and esterified forms, since strongly acidic media are employed in the colorimetric methods. Procedures which continue to be used widely are modifications of those described early by Dische. One is based upon reaction with cysteine (3) and the other with carbazole (4). One modification of the carbazole method, which gave a doubling in sensitivity, along with an increased stability in color and greater reproducibility, was reported in 1962 (5). Inclusion of borate into the assay medium was responsible for the enhancement of the method. In all the colorimetric methods, galacturonic acid is liberated during the assay by hydrolysis of polymeric pectin. An application of the carbazole method, after extraction of pectin from various fruits and vegetables, has been described (6), as has been an automated carbazole method for monitoring uronic acid levels in pectin fractions (7).

A more rapid and somewhat simpler procedure for uronic acid determination was described in 1973 (8). This method is advantageous for determining galacturonic acid in pectin, as interference by neutral sugars is reduced. This method is based on the color formation which accompanies the addition of m-hydroxybiphenyl to heated solutions of uronic acids in sulfuric acid/boric

acid. This assay has been applied to the determination of galacturonic acid in food pectins (9,10), and interference by neutral sugars was minimized (9). Another phenol, 3,5-dimethylphenol, has been found (11) to be more selective than m-hydroxydiphenyl when large amounts of neutral sugars are present in the sample. This method has recently been employed in studies of the degree of methylation of pectin in plant cell walls (12).

A procedure employing colloidal titration has been used for the determination of galacturonic acid in pectin, and indirectly, also for determining the degree of esterification (13). Samples are titrated with poly-N,N-dimethylallylammonium chloride, and a distinct flocculation occurs, the endpoint of which is determined by use of toluidine blue indicator. In a duplicate sample, ester methyl groups can be saponified, and total galacturonic acid determined; by difference, the degree of methyl esterification is calculated. The quantitation of this colloidal titration method is more precise with pectins of high degrees of polymerization. In another titrimetric method, total galacturonic acid and the degree of esterification is determined by copper-binding before and after saponification (14). The bound copper is determined by atomic absorption spectrometry. Application of this copper-binding approach to the analysis of cell-wall polysaccharides in many fruits and vegetables has been reported (15).

Decarboxylation with hydroiodic acid (16) was the basis for a procedure used in determining uronic acid levels in dietary fiber fractions (17). The carbon dioxide from decarboxylation was purified, trapped in a cell containing standard sodium hydroxide, and conductivity changes were measured using an Ingold electrode.

Studies comparing the distribution of free carboxyl groups in enzymatically and chemically de-esterified pectins are important because the gelling behavior of resulting products is a function of the method used. Enzymatically de-esterified pectins have a blockwise distribution of non-esterified galacturonic acid residues, and gel with calcium at higher degrees of esterification than do acid de-esterified pectins, which possess a more random distribution of free carboxyl groups. Free carboxyl distribution has been studied (18) by first esterifying by reaction with ethylene oxide (glycolation), and then treating the sample with a mixture of pectin enzymes. The glycolated fragments are unreactive toward these enzymes. Finally, the hydrolysis products are separated from the glycolated fragments by ion exchange chromatography, and after deglycolation, chain size is determined by gel filtration. An application of this approach in studies of orange-peel pectin has been reported (19).

Physical Methods. Infrared (IR), Raman, and nuclear magnetic resonance (NMR) spectroscopic methods have been applied to structural analysis of polysaccharides such as pectin. These applications have been reviewed (20), and reference IR spectra of pectic substances have been published (21). Quantitative IR has been used to estimate acid dissociation constants of polyuronides from the ratio of $-CO_2H$ to $-CO_2-$ as a function of pH (22). Also, by

methods to resolve neutral sugars are applied. The galactose (dideutero) which had been generated from galacturonic acid in pectin is distinguished from galactose (diprotio) naturally present in pectin by mass spectrometry. These GLC methods are characteristically very sensitive and efficient.

Liquid chromatographic procedures have been developed more recently, and some are quite effective for determining galacturonic acid in pectin. An automated anion-exchange chromatographic system (34) allows the separation of individual uronic acids, including galacturonic acid. Column effluents were sensitively analyzed for uronic acids by post-column reaction with orcinol and monitoring at 420 nm. More rapid HPLC approaches have been described. By using strong anion-exchange columns, 0.7M acetic acid mobile phase, and refractive index detection, galacturonic acid was separated from mannuronic and glucuronic acids in less than 15 minutes (35). Similar conditions were employed to separate oligogalacturonic acids of up to DP-8, and Bio-Gel P-2 has been used for the same purpose (36). In another report (37), strong anion-exchange HPLC also was used, with a mobile-phase consisting of boric acid/potassium hydroxide buffer, and post-column fluorometric detection (2-cyanoacetamide), allowing the resolution and sensitive detection of four uronic acids, including galacturonic acid. Polygalacturonic acid has been subjected to methanolysis (reaction with methanolic hydrogen chloride); multiple peaks result in HPLC chromatograms, due to the presence of $\alpha,\beta$-mixtures of the methyl glycosides (38). Although the procedure is not ideal for quantitation, it is useful for qualitative analysis of uronic acid composition in polysaccharides. In a recent report (39) polygalacturonic acid was subjected to both acid and polygalacturonase catalyzed hydrolysis. The hydrolyzates were analyzed by HPLC on a cation-exchange column of HPX-87-$H^+$, and galacturonic acid was eluted in 8.5 minutes. The advantages of enzyme over acid-catalyzed hydrolysis were apparent. The yield of monomer was greater, no monomer degradation products were present, and a far lesser quantity of oligogalacturonic acid chains were produced.

## Determination of Methyl, Acetyl, and Feruloyl Substitution in Pectin

Pectin consists mainly of polygalacturonate chains, and the carboxyl groups are significant determinants of its chemical and biological properties. In plant cell walls, more than 50% of the carboxyl groups are often esterified with methanol. The degree of esterification largely determines the ion-exchange, water-binding, cross-linking, and hydrogen-bonding capacities of pectin. Similarly, properties of pectin in cell walls are sometimes modified by low levels of hydroxyl esterification with acetyl groups. The distribution of acetyl groups in pectin is unknown, but in sugar beet, pear, and apricot pectin, acetyl levels approach 4%. In addition, alkali-labile ferulic acid groups are found in ester linkage to pectin; they are believed to be carried by arabinose and/or galactose residues on neutral side chains. This section will describe recent methods to determine pectin substitution with methyl, acetyl, and feruloyl groups.

Methyl Esters In Pectin. A titration method has been reported (40), in which methyl ester levels are calculated from the number of equivalents of standard sodium hydroxide required to neuturalize the pectin sample before and after saponification. The copper titration procedure described earlier for determination of galacturonic acid residues in pectin (15), is also used to determine methyl ester levels from the increase in copper-binding after hydrolysis of the esters. An accurate and sensitive colorimetric method (41) is rather time-consuming, but several samples can be run in parallel. Samples are saponified, the released methanol oxidized to formaldehyde, and the formaldehyde determined by spectrophometric assay (412nm) of its condensation product with pentane-2,4-dione.

GLC procedures are widely used for methyl ester determinations; after saponification with 0.5N base, methanol is measured by GLC on columns of Poropak Q at 120°C (42), or on Carbowax 1500 at 125°C (12). In the latter study, analyses were conducted on small samples of isolated plant cell wall preparations. In studies on the enzymatic incorporation of methyl groups from S -adenosyl-L-methionine into pectin (43), $^{14}$C-methyl-labelled substrate was used. The $^{14}$C-methanol, after release from pectin, was determined by GLC on Carbowax 300 using a radioactivity counting detector. The coupling of analytical pyrolysis to GLC has resulted in the detection of characteristic fragments from macromolecules, such as pectin. This topic has been reviewed (44), and correlations between degree of methyl esterification and intensity of some of the peaks have been made (45).

Acetyl and Feruloyl Esters in Pectin. A colorimetric method for determining degrees of acetylation in pectins from various sources (46), has been shown to be rapid and quite sensitive. Hydroxylamine is reactive toward both the methyl and acetyl esters in pectin, and ferric ion complexes with the resulting hydroxamic acids are red. The pectin complex is insoluble and removed by filtration; the intensity at 520nm in the soluble fraction, consisting of the ferric complex with acetohydroxamic acid, is a measure of acetyl content. The accuracy of the method was demonstrated in determinations of O-acetyl levels in standard per-acetylated polysaccharides. Another method (47) involves alkaline hydrolysis of the acetyl groups from pectin, followed by distillation of acetic acid and its titration with standard base.

In a study of the structure and functions of feruloylated pectins in primary cell walls in spinach, about one feruloyl group was found per sixty sugar residues (48). Ferulic acid was determined after alkaline hydrolysis by the Folin-Ciocalteu phenol reagent.

## Neutral Sugar Composition of Pectin

The neutral sugars, with the exception of L-rhamnose, are attached exclusively in sidechains, and include D-galactose, L-arabinose, D-xylose, and less frequently, D-glucose, D-mannose, L-fucose, 2-O-methyl-D-xylose, 2-O-methyl-D-fucose, and D-apoise. Whether

the sugars are determined by GLC or HPLC methods, it is essential that the polysaccharide first be hydrolyzed to its monomeric sugars. Acid-catalyzed hydrolytic methods are most often used, but the various linkages have different susceptibilities, as do the various sugars when released upon hydrolysis. These problems have been discussed, along with a review of the sugar GLC literature prior to 1973 (49). It was stated that "no one method of hydrolysis will necessarily cleave every linkage and give each component in quantitative yield." The Saeman hydrolysis (50), which employs 72% sulfuric acid, or 2N trifluoracetic acid (51) are used most often. When possible, enzymatic approaches in combination with acid hydrolysis are preferred for polysaccharide hydrolysis. After hydrolysis, the most widely used methods for sugar determination are based on GLC of suitably volatile derivatives. The derivatives in which the anomeric center is eliminated so that single peaks result are most effective.

Single-peak sugar derivatives which allow the resolution of sugar constituents in pectin include the trimethylsilylated methyloximes (52), acetylated aldononitriles (53), trimethylsilylated alditols (54), and acetylated alditols (51). A comprehensive review article on GLC of sugars has been published (26).

In studies of polysaccharides structure, the alditol acetate procedure remains the most widely used GLC procedure. The advent of high-resolution glass capillary columns has allowed very efficient separations. Recent applications of these columns to alditol acetate separations have been described (55-57). The alditol acetate procedure requires reduction of the sugars with sodium borohydride. After removal of boric acid, the sample is acetylated by conventional means. Various polar stationary phases have been used in GLC separation of alditol acetates, in both packed and capillary columns. A low-polarity phase was used in a report (54) which demonstrated the separation of trimethylsilylated alditols, and the neutral sugars in a hemicellulose sample were resolved.

A liquid chromatographic system has been applied in a study of monomer composition in cell-wall polysaccharide hydrolyzates (58). A strong-base anion-exchange column was eluted with a borate buffer step-gradient. Post-column reaction with orcinol allowed the sensitive determination of the sugars rhamnose, xylose, arabinose, glucose, galactose, and mannose. An improved two-step HPLC procedure for total resolution of the above neutral sugars has been published (59). On aminopropyl silica with acetonitrile-water as mobile phase, rhamnose is separated, but the pentoses xylose and arabinose are not well resolved, nor are the hexoses glucose, galactose, and mannose. These two multicomponent peaks are collected and re-chromatographed on Aminex HPX-87P, a heavy metal cation-exchanger in the lead form. Total resolution of the five monosaccharides results.

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Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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